Observations on Aspergilli in Santa Rosa National Park, Costa Rica

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Species of *Aspergillus* and their sexual states, *Emericella*, *Eurotium*, and *Neosartorya*, were isolated from soil or collected on natural substrata in Santa Rosa National Park in northwestern Costa Rica. Fungi were recovered by soil dilution plating, direct plating of soil on cyclosporine-containing media and direct plating on media used to recover osmotolerant and osmophilic fungi. We examined their distribution in soils collected at 1-km intervals along an 11-kilometer transect on the Santa Elena Peninsula. A large and diverse group of *Aspergillus* species were isolated. Nine or more species were found at more than half of the sampling points along the transect. Some of the same species described from Costa Rica by Raper and Fennell were also recovered in this study.

In addition to morphological identification, representatives of all *Aspergillus* species were subjected to DNA sequencing using the ITS1-5.8S-ITS2 regions and the 28S D1-D2 regions. Sequencing selected regions of ribosomal DNA were an effective technique in the identification of *Aspergillus*. In 29 out of the 35 isolates sequenced, the molecular data agreed with the macro- and micromorphology at least at the group level, and in 12 of these cases, to the species level. Four species could only be recognized by reference to the molecular data. In a few cases, especially with members of the *A. ochraceus* group, molecular data appeared to be misleading and was in direct conflict with our morphological evaluations.

Fallen fruits of *Enterlobium cyclocarpum* and *Guazuma ulmifolia* were infested with viable sclerotia of at least 4 species of *Aspergillus*. *Emericella variecolor*, infrequently cited in the literature, was abundant on fruits of *E. cyclocarpum* as well as in soils. *Aspergillus aeneus* occurred as masses of Hülle cells on litter fragments on the forest floor, in addition to being isolated from soils.

Key words: *Aspergillus*, dry forests, ITS, ribosomal large subunit, sclerotia, soil fungi.

Introduction

This report presents information on species of *Aspergillus* and their *Emericella*, *Eurotium* and *Neosartorya* sexual states, isolated from soils or collected on natural substrata in Santa Rosa National Park in northwestern

Costa Rica. Few studies have focused on *Aspergillus* in Costa Rica. Thirteen species were reported in a check list of Costa Rica fungi (Covington, 1980). The majority of those reports were based on two studies of Panamanian and Costa Rican soil fungi where isolates of agricultural and forest soils from southern Costa Rica were enumerated (Farrow, 1954; Goos, 1960). The potential diversity of Aspergilli in Costa Rica and Central America was revealed by Raper and Fennell their monograph of *Aspergillus* (1965) when they described 12 new species from soil isolates. Subsequently, during a monographic revision of the black Aspergilli, *A. helicothrix* Al-Musallam, was reported from Costa Rica (Al-Musallam, 1980).

During the course of a cooperative project with the Instituto Nacional de Biodiversidad (INBio), we made numerous collections of soil and litter fungi in the Guanacaste Conservation Area (GCA), including Santa Rosa National Park. The high frequencies of Aspergilli encountered in soils and the numerous observations of Aspergilli on fruits and plant debris, especially in the dry forests, intrigued us. We investigated the distribution of *Aspergillus* species along an 11-km transect on the Santa Elena Peninsula of Santa Rosa National Park to better understand which species might be widespread in the soils of a typical tropical dry forest. We identified these *Aspergillus* isolates using classical morphological identification (Raper and Fennell, 1965; Al-Musallam, 1980; Christensen, 1982; Christensen and States, 1982). In addition, we extracted, amplified and sequenced the D1-D2 regions of the large subunit (LSU) ribosomal RNA (rDNA) and the internal transcribed spacer (ITS1-5.8S-ITS2) region. These wild type sequences were analyzed and compared to sequences derived from authenticated *Aspergillus* strains, including type specimens. This set of sequences was generated by S.W. Peterson, U.S.D.A., Peoria, IL, U.S.A. who analyzed the sequences of the D1-D2 regions from 215 known taxa of *Aspergillus* and made them available through GenBank (Peterson, 2000).

Aspergilli are important inhabitants of seeds and fruits of domesticated plants and their sclerotia are an adaptation to the seed habitat (Wicklow, 1985), but little is known about the natural history of wild seed- and fruit-inhabiting Aspergilli. We, therefore, report on specific and widespread infestations of fallen fruits of two major tree species of the tropical dry forest by cleistothecia and sclerotia of Aspergilli.

Materials and methods

Study Area

The GCA encompasses three National Parks; Santa Rosa, Guanacaste, and Rincón de la Vieja, as well as Horizontes Experimental Station and the

Wildlife Refuge of Bahía Junquillal. The geology and the characteristics of the tropical dry forests of the region have been summarized previously (Janzen, 1983). A portion of the Santa Elena Peninsula lies within Sector Murciélago of Santa Rosa National Park and is one of the most arid regions of the GCA. Dry forests of the region are characterized by harsh dry seasons of approximately 6 months and wet seasons with 1-3 m of rainfall. A large proportion of the canopy trees are deciduous or semi-deciduous. As a result, in the dry season, sunlight often penetrates to the forest floor and the litter and upper soil horizons are desiccated to the point that decomposition may cease.

Frequency and identification of Aspergilli

During June, 1997, soil samples were collected in the Santa Elena Peninsula along the Murciélago road at 1-km intervals starting at 0 km at the beach-forest transition at Playa Blanca and proceeding eastward until kilometer 10. At each kilometer interval, 2 soil samples were collected, 1 from each side of the one lane road for a total of 22 soil samples. Soils were collected from the first few cm below the litter layer with a soil-immersed knife and stored in Tyvek soil collection bags. The soil samples were transported at ambient temperatures to Merck Research Laboratories, Rahway, NJ and stored at 4 C until processed. The 22 soil samples were processed using 3 qualitative techniques to isolate Aspergilli:

1. *Direct plating*. A small amount of soil (approximately 0.1-0.2g) is sprinkled onto MYE (malt extract, 1%; yeast extract, 0.2%) agar plates with 10 mg/L cyclosporine to retard fast-growing fungi. One plate was examined per sample.
2. *Direct plating*. A small amount of soil (approximately 0.1-0.2g) was sprinkled onto DG-18 (Oxoid) agar plates to select for osmotolerant and osmophilic fungi. One plate was examined per sample.
3. *Soil suspensions*. One ml of 1/500, 1/2500 and 1/5000 dilutions (w/w) in 0.2% carboxymethylcellulose were spread with a bent glass rod onto DG-18 agar plates. All plates were incubated at 23 C at 80% relative humidity and a 12 h light/dark cycle. One plate was examined per each dilution per each soil sample.

In total, 5 plates for each sampling point and 110 plates for the length of the transect were examined.

Streptomycin sulfate and chlortetracycline were added at 50 mg/l to all isolation media. After 7 days incubation, the plates were examined by eye and with a dissecting microscope every 2-3 days for up to 28 days. Morphologically unique colonies were transferred to MYE agar slants and

incubated until substantial growth occurred. For identification, cultures of *Aspergillus* species were transferred to Blakeslee's malt-extract agar, DG-18 and Czapek's sucrose agar (Raper and Fennell, 1965; Pitt, 1979) for identification. Nomenclature for species groups follows that designated by Raper and Fennell (1965) because their classification system is integrated into the only comprehensive identification guide for *Aspergillus*. For correlations with molecular analyses we referred to an updated infrageneric classification for *Aspergillus* (Gams *et al.*, 1985).

Representative strains described herein were preserved in 10% glycerol at -80 C at Merck Research Laboratories, Rahway, NJ, U.S.A.

DNA extraction and PCR

28S rDNA: Forty-two cultures deemed to be representative of the morphological groups encountered were grown on MYE plates with a 2-inch square of cellophane (Flexel Corp, Indiana) for 10-14 days. Ribosomal DNA was extracted from the fungal mycelia using Invitrogen Easy-DNA kit (Carlsbad, CA, U.S.A., kit 45-0424) following "Protocol #3-Small amounts of cells, tissues, or plant leaves". Primers used to amplify the large subunit ribosomal DNA were D1 and D2 (Peterson, 2000) supplied by GibcoBRL, Gaithersburg, MD, U.S.A. or Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A. The PCR products were extracted and purified from agarose using QIAquick gel extraction kit (Qiagen, Santa Clarita, CA, U.S.A., kit 28704).

For the ITS regions, the procedures followed for culture growth, DNA extraction and amplification of the ITS1-5.8S-ITS2, are described previously (Bills *et al.*, 1999). The primers used to amplify the internal transcribed sequences were ITS1F (Gardes and Bruns, 1993) and ITS4 (White *et al.*, 1990).

DNA sequencing and phylogenetic analysis

Sequencing reactions used ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit protocol performed on a PE Applied Biosystems model ABI 377 sequencer. Sequences were analyzed and contigs assembled with Factura and AutoAssembler software (PE Applied Biosystems). Resulting sequences were searched in GenBank using the FASTA protocol. Sequence alignment was performed manually. In the case of the internal transcribed spacers, the final rearrangement among groups of sequences was performed using CLUSTALW software. The phylogenetic and cladistic analysis of the aligned sequences was performed using the maximum-parsimony analysis with the branch-and-bound algorithm of the PAUP 3.1.1 software (Swofford, 1993).

Results and discussion

Aspergilli recovered from transects

One hundred and forty-four isolates of *Aspergillus*, representing 42 colony types (Table 1) were recovered from soils collected along an 11-kilometer transect in a tropical dry forest in the Santa Elena Peninsula. Based on morphological and microscopic characteristics and molecular analyses, 29 distinct species or groups were established (Table 2). Strains of *A. niger* and *A. ochraceus* groups, and *A. tamarii*, *A. flavipes*, *A. japonicus*, *Emericella variecolor*, *A. pulvinus*, *A. puniceus* and *A. auricomis* were the most widespread Aspergilli along the 11-km transect (Table 2). Species of the *A. niger* group and *A. nidulans* group were the most commonly isolated groups in the 11-km transect. No correlation was obvious between sampling points along the transect and preference of *Aspergillus* species or groups. The highest numbers of species or groups (12) were recovered from kilometers 0, 3 and 6, while the lowest numbers (5) were recovered from kilometer 7. With classical identification, it was possible to classify most of the strains into a particular morphological group (Raper and Fennell, 1965), and the majority of types could be identified to species or species complex. This level of species richness is comparable to other studies of Aspergilli in tropical and subtropical forests (Christensen and Tuthill, 1985). Among the 16 species noted in Costa Rica by Raper and Fennell (1965), we recovered *A. aeneus*, *A. eburneo-cremeus*, *A. japonicus*, *A. niger*, *A. pulvinus* and *A. puniceus*.

Identification of Aspergilli using sequencing of rDNA regions

DNA extractions or PCR amplifications failed or sequences were unreadable in a significant portion of the 42 morphological types. We obtained sequences of the D1-D2 regions of the large subunit ribosomal RNA from 29 of 42 strains. The sequences of the ITS1-5.8S-ITS2 region were obtained from 24 isolates. Therefore, for 17 strains we obtained both the sequences of the D1-D2 region as well as the ITS1-5.8S-ITS2, and for 36 of the 42 total types at least one of the two regions was sequenced (Table 1). The sequences were compared with the data available in GenBank using FASTA. The sequences giving the best matches in each case were aligned manually and the percentage of similarity were calculated based on the number of positions conserved.

As a rule, we observed a reasonable agreement between the sequence data and the morphological classification (Table 1). In 29 out of the 36 isolates the molecular data agreed with the morphology at least at the group level, and in 14 of these cases, at the species level. Both rDNA regions sequenced showed similar capabilities to be used for the identification of wild isolates. Thus, a

Table 1. Morphological and molecular identification of 41 *Aspergillus* isolates. Morphological identifications are in two levels of resolution, the species group of Raper and Fennell (1965), followed by species, species complex, or distinguishing morphological features. Molecular identifications were derived from FASTA searches of GenBank using the 28s LSU region and the ITS1-5.8s-ITS4 region. ND indicates DNA extraction or PCR amplifications failed.

		Identification		
Strain (ASP)	Group[2]	Species, morphology	% similarity to GenBank accessions[1]	
			28s LSU D1-D2 region	ITS1-5.8s-ITS4 region
1	Glaucus	*Eurotium amstelodami*	*Eurotium amstelodami* NRRL 89 U29549 (99) *Eurotium chevalieri* NRRL 79 U29547 (99)	*Eurotium rubrum* u18357 (98) *Aspergillus restrictus* l76776 (98)
2	Ornatus	Orange to black sclerotia, strictly uniseriate conidial heads	*Aspergillus caelatus* NRRL 443 AF004931 (97) *Aspergillus caelatus* NRRL 25528 AF004930 (97)	*Aspergillus flavus* ABOO8414 (99)
3	Nidulans	*Emericella variecolor*	ND	ND
4	Ochraceus	*Aspergillus auricomis*	*Aspergillus kanagawaensis* NRRL 2161 U29808 (93) *Aspergillus sparsus* NRRL 1933 U1791028S (91)	ND
5	Flavus		*Aspergillus nomius* NRRL25585 AF027861 (99) *Aspergillus nomius* NRRL13137 AF027860 (99)	ND
6	Flavus	*Aspergillus tamarii*	ND	*Aspergillus tamarii* ABOO8420 (81) *Aspergillus flavus* ABOO8414 (80)
7	Flavus	*Aspergillus tamarii* with sclerotia	ND	*Aspergillus tamarii* ABOO8420 (99) *Aspergillus flavus* ABOO8414 (97) *Aspergillus parasiticus* ABOO8418 (97)

[1]Pecentage similarities in parentheses are based on similarity to GenBank accession numbers.
[2]Groups correspond to species groups in Raper and Fenell (1965).

Table 1. (continued).

Strain (ASP)	Group[2]	Species, morphology	Identification: % similarity to GenBank accessions[1]	
			28s LSU D1-D2 region	ITS1-5.8s-ITS4 region
8	Flavus	*Aspergillus parasiticus*, white sclerotia	*Aspergillus tamarii* NRRL 20818 AF004929 (99) *Aspergillus tamarii* NRRL 26066 AF004932 (99)	*Aspergillus tamarii* ABOO8420 (99) *Aspergillus flavus* ABOO8414 (97) *Aspergillus parasiticus* ABOO8418 (97)
9	Flavus	*Aspergillus avenaceus*	*Aspergillus tamarii* NRRL 20818 AF004929 (99) *Aspergillus tamarii* NRRL 26066 AF004932 (99)	*Aspergillus flavus* ABOO8414 (98) *Aspergillus parasiticus* ABOO8418 (98)
10	Ochraceus	Small yellow sclerotia	ND	*Aspergillus parasiticus* ABOO8418 (84) *Aspergillus ellipticus* u65310 (84) *Aspergillus flavus* ABOO8414 (84) *Emericella heterothallica* L76746 (84)
11	Nidulans	Yellow masses of of Hülle cells	*Aspergillus multicolor* NRRL 4775 U29835 (98) *Emericella striata* NRRL4699 U29845 (98	*Emericella nidulans* L76746 (96) *Aspergillus versicolor* L76745 (94)
12	Ustus	*Aspergillus puniceus* (=ASP30)	ND	*Emericella heterothallica* L76743 (94) *Aspergillus ustus* L76744 (93)
13	Flavipes	*Aspergillus flavipes*	ND	*Fennellia flavipes* L76755 (97)
14	Versicolor	*Aspergillus pulvinus*	*Aspergillus pulvinus* NRRL 5078 U20822 (99) *Aspergillus terreus* NRRL 260 U28839 (96)	*Eupenicillium javanicum* u18358 (89) *Eupenicillium ehrlichii* AFO33432 (89)
15	Nidulans	*Aspergillus aeneus*	*Fennellia flavipes* NRRL 302 U28853 (99) *Fennellia flavipes* NRRL 295 U28852 (99)	*Aspergillus versicolor* L76745 (87) *Emericella nidulans* L76746 (87)
16	Flavipes	*Aspergillus flavipes*	ND	ND

Table 1. (continued).

Strain (ASP)	Group[2]	Identification: Species, morphology	% similarity to GenBank accessions[1]: 28s LSU D1-D2 region	ITS1-5.8s-ITS4 region
17	Niger	Large sclerotia	*Aspergillus eburneo-cremeus* NRRL 4773 U29834 (100) *Aspergillus aeneus* NRRL 4769 U29826(98	ND
18	Niger	Scant sporulation	*Aspergillus phoenicis* NRRL 5121 U28818 (100)	ND
		Pink sclerotia	*Aspergillus niger* NRRL 326 U28812 (99)	
19	Niger	Black soluble pigment	ND	ND
20	Niger	*Aspergillus japonicus*	ND	*Aspergillus aculeatus* u65309 (97)
21	Niger	Moderate conidiogenesis, large sclerotia	ND	ND
22	Ochraceus	Reddish mycelium	*Aspergillus avenaceus* isolate NRRL 4517 U15500 (96)	ND
		Cream colored sclerotia	*Aspergillus clavato-flavus* NRRL 5113 U28912 (96)	
23	Cremeus	*Aspergillus flaschentraegeri*	*Aspergillus flaschentraegeri* NRRL 5042 U15504 (99) *Aspergillus dimorphicus* NRRL 3650 U15491 (98)	*Fennellia flavipes* L76745 (89) *Aspergillus tamarii* ABOO8420 (88) *Aspergillus parasiticus* ABOO8418 (88)
24	Ochraceus	Sclerotia absent, abundant white aerial mycelium	*Aspergillus kanagawaensis* NRRL 2161 U29808 (97) *Aspergillus anthodesmis* NRRL 22884 U17916 (97)	ND
25	Nidulans	*Emericella variecolor*	*Emericella astellata* NRRL 2396 U29847(99 *Emericella variecolor* NRRL 1954 U29846(98	*Aspergillus versicolor* L76745 (95)

Table 1. (continued).

Strain (ASP)	Identification			
	Group[2]	Species, morphology	% similarity to GenBank accessions[1]	
			28s LSU D1-D2 region	ITS1-5.8s-ITS4 region
26	Flavipes	*Aspergillus flavipes*	ND	ND
27	Versicolor	*Aspergillus sydowii*	*Aspergillus caesiellus* NRRL a-14879 U29651 (100) *Aspergillus ustus* NRRL 1974 U29786 (98)	*Aspergillus versicolor* L76745 (97)
28	Fumigatus	*Aspergillus fumigatus*	*Neosartorya fischeri* NRRL 179 U28467 (99) *Neosartorya fennelliae* NRRL 5534 U28470 (99)	ND
29	Niger	*Aspergillus niger* with light pink sclerotia	*Aspergillus phoenicis* NRRL 5121 U28818 (100) *Aspergillus niger* NRRL 326 U28812 (99)	ND
30	Ustus	*Aspergillus puniceus*	*Aspergillus puniceus* NRRL 1852 U29788 (99) *Aspergillus ustus* NRRL 1974 U29786 (99)	*Emericella heterothallica* L76745 (94) *Aspergillus ustus* L76744 (93)
31	Terreus	*Aspergillus terreus*	*Aspergillus terreus* NRRL 260 U28839 (99)	ND
32	Niger	*Aspergillus niger*	ND	ND
33	Ochraceus	Sclerotia absent, yellow rough conidiophores	*Aspergillus clavato-flavus* NRRL 5113 U28912 (95) *Aspergillus brunneo-uniseriatus* NRRL 4273 U29823 (94)	*Aspergillus ellipticus* U65310 (87)
34	Nidulans	Dense masses of Hülle cells, no conidiophores	*Aspergillus eburneo-cremeus* NRRL 4773 U29834 (100) *Aspergillus aeneus* NRRL 4769 U29826(98	*Aspergillus versicolor* L76745 (87) *Emericella nidulans* L76746 (87)
35	Nidulans	*Aspergillus nidulans*	*Emericella acristata* NRRL 2394 U29857(99 *Emericella echinulata* NRRL 2395 U29842(99	ND

Table 1. (continued).

Strain (ASP)	Group[2]	Species, morphology	Identification	
			% similarity to GenBank accessions[1]	
			28s LSU D1-D2 region	ITS1-5.8s-ITS4 region
36	Nidulans	*Emericella variecolor*	*Emericella variecolor* NRRL 1954 U29846 (100) *Emericella astellata* NRRL 2396 U29847 (99)	ND
37	Nidulans	Dense masses of Hülle cells, no conidiophores	*Aspergillus eburneo-cremeus* NRRL 4773 U29834 (100) *Aspergillus aeneus* NRRL 4769 U29826 (98)	*Aspergillus versicolor* L76745 (87) *Emericella nidulans* L76746 (87)
38	Nidulans	*Aspergillus aeneus*	*Aspergillus elongatus* NRRL 5176 U29799 (97) *Aspergillus raperi* NRRL 2640 U29820 (97)	ND
39	Not determined	Non-*Aspergillus*	*Geosmithia cylindrospora* strain NRRL 2673 AF033386 (91) *Geosmithia argillacea* NRRL 5177 AF033389 (89)	*Penicillium trachysporium* L14516 (97) *Talaromyces gossypii* L14523 (96)
40	Versicolor	*Aspergillus cf. versicolor* Long yellow conidiophores	*Aspergillus ustus* NRRL 1974 U29786 (98) *Aspergillus puniceus* NRRL 1852 U29788 (98)	*Aspergillus versicolor* L76745 (98) *Emericella nidulans* L76746 (96)
41	Nidulans		*Emericella astellata* NRRL 2396 U29847 (99) *Emericella variecolor* NRRL 1954 U29846 (98)	*Aspergillus versicolor* L76745 (95) *Emericella nidulans* L76746 (94)
42	Ustus		ND	*Emericella heterothallica* L76743 (95) *Aspergillus ustus* L76744 (93)

positive result was obtained in 70% of the isolates, for both types of sequences. The D1-D2 region allowed the identification at the species level in a slightly higher number of cases, compared to the ITS region (40% vs. 30%). This is probably related with the fact that the number of sequences from the D1-D2 region available in Genbank was higher than for the ITS region.

Sequencing of the rDNA regions allowed us to identify *A. eburneo-cremeus* (ASP 34, 37) and *A. aeneus* (ASP 15), which produce large masses of Hülle cells in plate cultures, and *A. flaschentraegeri* (ASP 23), *A. tamarii* (ASP 8) and *A. phoenicis* (ASP 29) whereas we were unable to recognize these fungi using morphological methods. The isolate ASP 39, which produced few sporulating structures, was identified as a *Talaromyces* sp. by molecular data (Figs. 1, 2). Subsequently it was confirmed that the fungus was not an *Aspergillus* by morphology and it was eliminated from the study.

The most evident disagreements between morphological and molecular data were found in the following cases:

1. ASP 4 and 24, *A. ochraceus* group by morphology, closer to the *A. cervinus* and *A. sparsus* groups by sequencing of the D1-D2 region.
2. ASP 10, *A. ochraceus* group by morphology, no good matches by sequencing analysis.
3. ASP 22, *A. ochraceus* group by morphology, closer to the *A. flavus* group by sequencing of the D1-D2 region.
4. ASP 33, *A. ochraceus* group by morphology, closer to the *A. flavus* group by sequencing of the D1-D2 region and to the *A. niger* group by ITS region.
5. ASP 17, *A. niger* group with sclerotia by morphology, closer to the *A. nidulans* group by sequencing of the D1-D2 region.

On the other hand in three cases, the analyses of both regions appeared to contradict each other:

1. ASP 14 and ASP 23, in *A. versicolor* and *A. cremeus* groups respectively by morphology, gave a very good match with members of those same groups by sequencing of the D1-D2 region, whereas with the ITS region the best matches (below 90%) were with *Eupenicillium* spp. (for ASP 14) and with the *A. flavipes* and *A. flavus* group (for ASP 23). In these cases, the low percentages of similarity found with the ITS sequences would explain the apparent contradiction.
2. ASP 15, in *A. nidulans* group, specifically *A. aeneus*, by morphology, matched with the same group based on the ITS sequences (although with a low percentage of similarity), but it was closer to the *A. flavipes* group, according to the D1-D2 sequence.

Table 2. Occurrence of 29 *Aspergillus* species or morphological types recovered from soil in a dry tropical forest along an 11-kilometer transect. Identifications based on combined morphological and molecular data in Table 1.

Morphological species or species group; Strain number(s)	Kilometer										
	0	1	2	3	4	5	6	7	8	9	10
Aspergillus niger V. Tiegh ASP 32 (no sclerotia)	•	•	•	•	•	•	•	•	•	•	•
A. ochraceus group ASP 22 (pink sclerotia)	•	-	•	•	•	•	•	•	•	•	•
A. tamarii Kita ASP 6, 7	•	•	•	•	•	•	•	-	•	•	-
A. flavipes Thom and Church ASP 13, 16, 26	-	-	-	•	•	•	•	•	•	•	•
A. japonicus Saito ASP 20	•	-	•	•	•	•	•	-	•	-	-
A. auricomis (Guéguen) Saito ASP 4, 10	•	-	-	•	•	-	•	•	-	•	-
Emericella variecolor Berk. and Broome ASP 3, 25, 36, 41	•	-	•	-	-	-	•	-	•	•	-
A. pulvinus Kwon and Fennell ASP 14	-	-	-	-	•	•	•	-	•	-	•
A. puniceus Kwon and Fennell ASP 12, 30	•	•	•	•	•	-	-	-	-	-	-
A. niger group (heavy sporulation, large sclerotia) ASP 17	-	•	-	-	•	-	-	-	•	•	-
A. flaschentraegeri Stolk ASP 23	-	-	-	•	-	-	-	-	•	•	-
A. niger group (moderate sporulation, large sclerotia) ASP 21	-	-	-	•	•	-	-	-	-	-	•
Eurotium amstelodami Mangin ASP 1	-	•	-	-	-	-	•	-	-	-	-
A. eburneo-cremeus Sappa ASP 34, 37			•							•	
A. niger group (dense sclerotia) ASP 18	-	•	-	•	-	-	-	-	-	-	-
A. ochraceus group ASP 33	-	-	•	-	-	•	-	-	-	-	-
A. aeneus Sappa ASP 15, 38	•	-		-	-	-	-	-	-		-
A. flavus Link ASP 5	-	-	-	-	-	•	-	-	-	-	-

• presence of a particular *Aspergillus* species or morphological type.

Table 2. (continued).

Morphological species or species group; Strain number(s)	Kilometer										
	0	1	2	3	4	5	6	7	8	9	10
A. flavus group ASP 2	•	-	-	-	-	-	-	-	-	•	-
A. cf. avenaceus Smith ASP 9	•	-	-	-	-	-	-	-	-	-	-
A. fumigatus Fresenius ASP 28	-	-	-	-	-	-	•	-	-	-	-
A. sydowii Thom and Church ASP 27	-	-	-	-	-	-	-	-	•	-	-
A. terreus Thom ASP 31	-	-	-	-	-	•	-	-	-	-	-
A. versicolor group ASP 11	-	•	-	-	-	-	-	-	-	-	-
A. niger group (black pigment) ASP 19	-	-	-	•	-	-	-	-	-	-	-
Emericella nidulans Vuilm. ASP 35	•	-	-	-	-	-	-	-	-	-	-
A. cf. versicolor ASP 40	-	-	-	-	-	-	•	-	-	-	-
A. phoenicis (Corda) Thom ASP29	-	-	-	-	-	-	•	-	-	-	-
A. ochraceus group ASP 24	-	-	-	-	-	-	-	•	-	-	-

From these results it could be concluded that none of the strains belonging to the *A. ochraceus* group could be properly identified using rDNA sequencing. On the contrary, members of the *A. flavus*, *A. glaucus*, *A. nidulans*, *A. ustus*, *A. versicolor* and *A. niger* groups, were usually properly identified to the group level by sequencing of either of the two rDNA regions. The only member sequenced of the *A. flavipes* group was correctly identified based on ITS sequencing; likewise members of the *A. fumigatus* and *A. terreus* groups were properly identified by sequencing of the D1-D2 region.

It is especially intriguing why strains ASP 17 and ASP 15 behaved abnormally, when the rest of strains from the same groups gave good matches with the expected sequences. In any case, the finding of a significant number of cases in which the identification suggested by the molecular data is misleading compared to the morphological data, should alert about the risks of giving absolute confidence to sequence data for identification purposes, at least for these fungal groups.

Figure 1 shows the cladograms generated with the ITS1-5.8S-ITS2 sequences obtained from the wild isolates, plus some sequences retrieved from

Genbank based on the best matches obtained through FASTA. All relationships shown in Table 1 are confirmed after the phylogenetic analysis. This was true not only for those isolates in which a good correlation was found between morphology and molecular data, but also for those in which a lack of consistency was detected.

In general terms, the D1-D2 region tree (Fig. 2) was consistent with the data from Peterson (2000). In both cladograms, the sequences corresponding to the *A. nidulans*, *A. ustus* and *A. versicolor* groups (Subgenus *Nidulantes*, Section *Nidulantes*) are grouped in a cluster. This branch was supported by a good bootstrap index in the tree obtained with the ITS sequences, but it is not as robust in the one generated with the D1–D2 region. In the tree obtained with the ITS regions (Fig. 1), the sequences corresponding to the *A. ustus* group appeared separated in a subclade within that cluster, whereas species from the *A. nidulans* and the *A. versicolor* groups are mixed in the same clade. The *A. aeneus* strains were rooted in that main branch. In the tree generated from the D1–D2 region (Fig. 2), the cluster containing these species is not as structured, but still the *A. aeneus* strains are grouped apart from the rest. Section *Flavi* also formed an independent cluster in both trees, supported by a good bootstrapping index. Other sections containing a lower number of members appeared also as independent clusters, supported by high bootstrap indexes. This was the case of sections *Flavipedes*, *Fumigati* and *Cremei*. Section *Nigri* appeared to be divided in two separate branches in both trees. The *A. pulvinus* sequences were clustered together with the *A. terreus* group, instead of being within the *A. versicolor* group. However, *A. pulvinus* was transferred from section *Versicolores* to section *Cremei* based on the 28S sequence (Peterson, 1995), and a more comprehensive study by the same author (Peterson, 2000) revealed that the situation of that species is relatively intermediate between sections *Cremei* and *Terrei*. Consistent with these observations, the two *A. pulvinus* sequences appear in the same branch as section *Terrei* (Fig. 2). The sections *Aspergillus/Restricti*, which are known to be sibling groups (Peterson, 2000), appeared also intermingled in our trees.

Aspergilli on fruits in the dry forest

During June, 1997, we observed that fallen fruits of two common trees, *Enterlobium cyclocarpum* (*Mimosaceae*) and *Guazuma ulmifolia* (*Sterculiaceae*) in and around the administration area of Santa Rosa National Park were infested with abundant masses of sclerotia or cleistothecia of Aspergilli. Masses of sclerotia of *Apergillus ochraceus* and *A. auricomis*, as well as scattered sclerotia of *A. niger*, colonized the pericarp surfaces of *E. cyclocarpum* (Fig. 3). Cleistothecia of *Emericella variecolor* also were

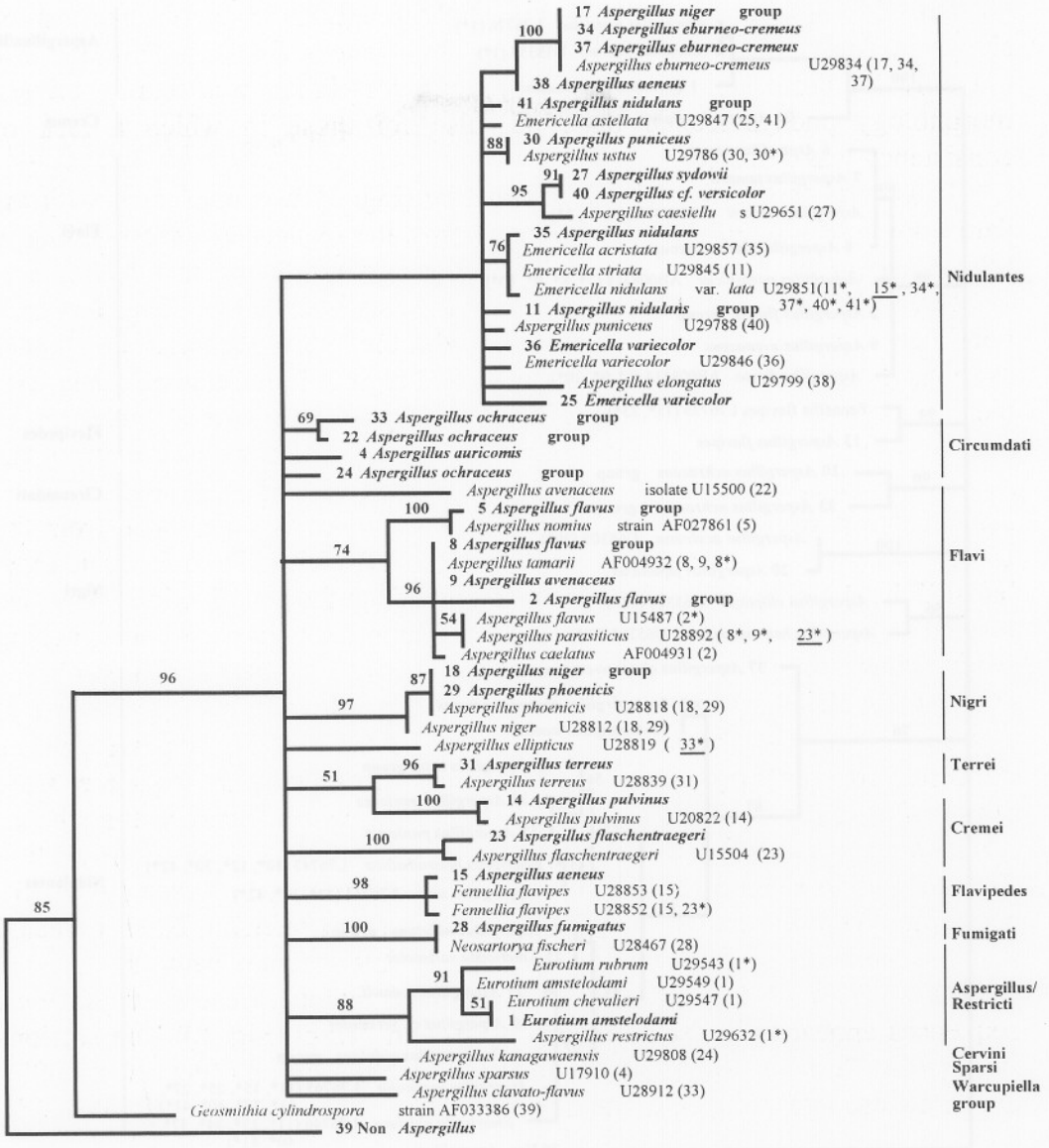

Fig. 1. Most parsimonious phylogenetic tree generated from a branch and bound algorithm in PAUP 3.1.1 from alignments of the sequences of the ITS1-5.8S-ITS2. The numbers above branches indicate the bootstrap percentage from 1000 bootstrap replicates. The trees has been rooted in a non-*Aspergillus* isolate (ASP 39). The numbers after the sequences retrieved from GenBank refer to the codes of the wild isolates presenting the best match with that sequence. Those numbers marked with asterisk refer to the best match for the ITSs sequence. *Aspergillus* sections, as established by Peterson (2000), are indicated to the right of the trees.

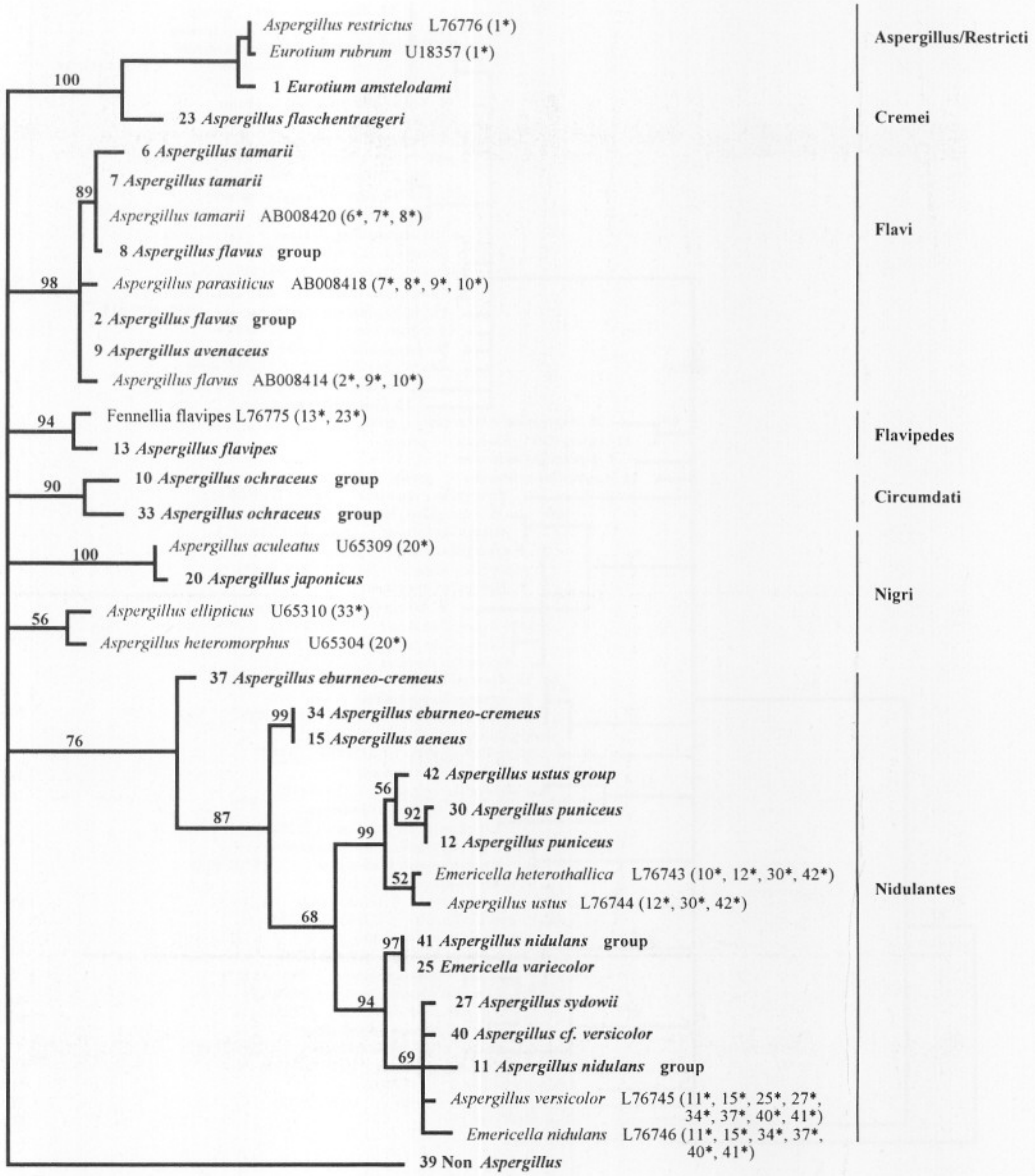

Fig. 2. Most parsimonious phylogenetic tree generated from a branch and bound algorithm in PAUP 3.1.1 from alignments of the sequences of the D1-D2 regions of the 28S rDNA. The numbers above branches indicate the bootstrap percentage from 1000 bootstrap replicates. The tree has been rooted in a non-*Aspergillus* isolate (ASP 39). The numbers after the sequences retrieved from GenBank refer to the codes of the wild isolates presenting the best match with that sequence. Those numbers marked with asterisk refer to the best match for the ITSs sequence, those without asterisk to the best match for the 28S. The numbers underlined indicate isolates that having the best match with the control strains are clustered in a different cluster. *Aspergillus* sections, as established by Peterson (2000), are indicated to the right of the trees.

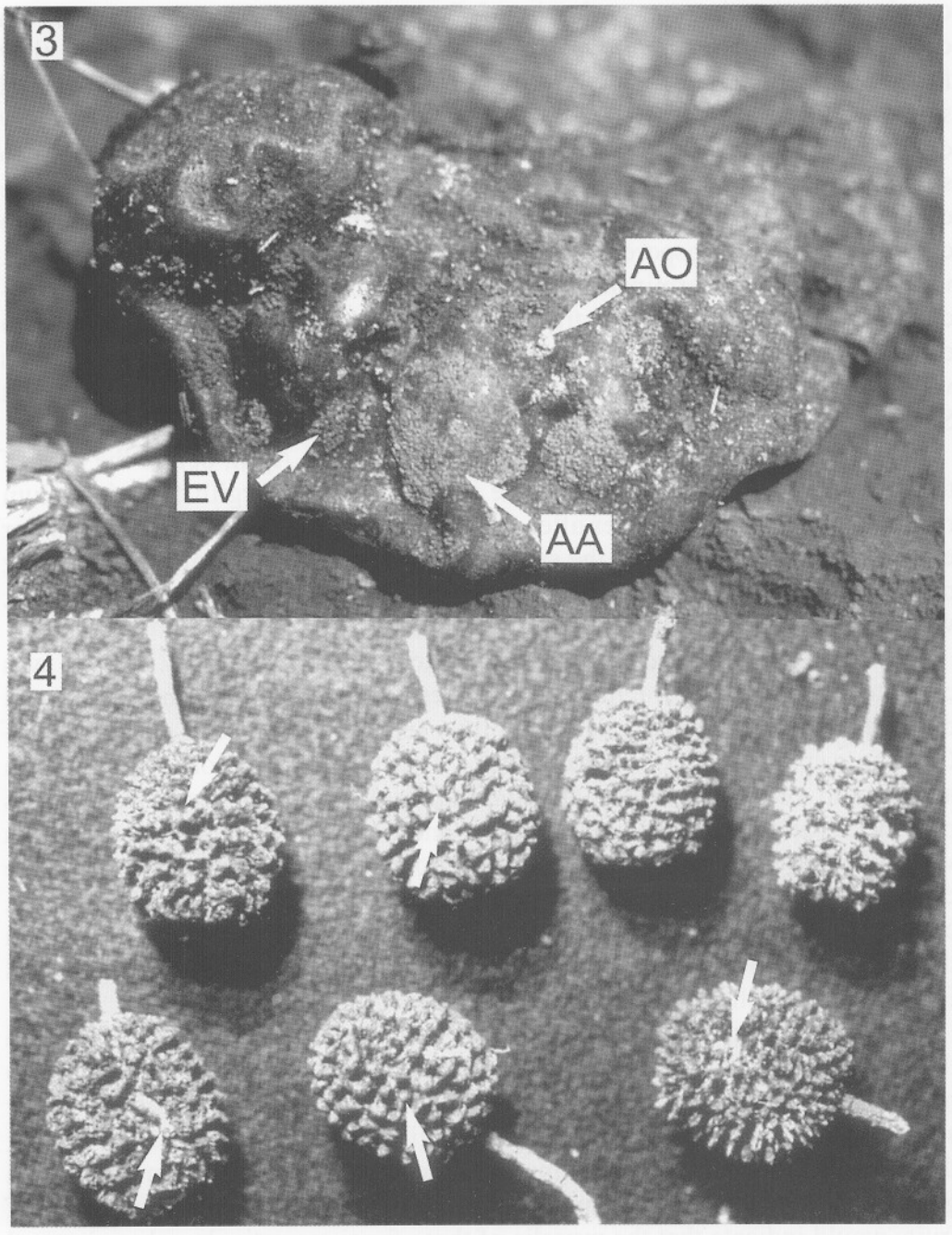

Fig. 3. Fruit of *Enterlobium cyclocarpum* infested with sclerotia of *A. ochraceus* (AO) and *A. auricomis* (AA) and cleistothecia of *Emericella variecolor* (EV). **Fig. 4.** Fruits of *Guazuma ulmifolia* with scattered sclerotia (arrowed) of *A. ochraceus* and *A. niger* inside seed locules. Bars =1 cm.

consistently abundant on pericarps of *E. cyclocarpum* (Fig. 3). Sclerotia on dehisced fruits of *G. ulmifolia* were more difficult to observe because they formed inside the deeply dissected empty seed locules (Fig. 4), but scattered sclerotia of *A. niger*, *A. ochraceus* and *A. auricomis* were consistently observed. For both tree species, virtually 100% of the fruits were infested to some degree with *Aspergillus* sclerotia and/or cleistothecia. Cultures could be established easily from any of these sclerotial types by transfer of sclerotia to culture media. Ascospores from fresh field-collected cleistothecia of *E. variecolor* did not germinate on standard mycological media, e.g. cornmeal-dextrose agar or malt-yeast extract agar, but Hülle cells germinated readily to establish cultures. *Aspergillus sclerotiorum* Huber, possibly the same fungus we have identified as *A. ochraceus*, was previously noted on "rotten fruits" at Santa Rosa Biological Station (Mercado-Sierra *et al.*, 1997).

Mature fruits of both these trees drop in March to April, before the start of the rainy season. Fruits likely become infested with Aspergilli from inoculum reservoirs in the soil and litter after commencement of the first rains in May or early June. In January 1998, the same sites were revisited during the beginning of the dry season. Most of the *G. ulmifolia* fruits had decomposed entirely, while only scant remnants of *E. cyclocarpum* fruits remained.

On two or three occasions in Santa Rosa National Park, *A. aeneus* was collected on dead twig fragments or petioles in the litter layer where it formed discrete patches of Hülle cells on the litter (e.g. strain GB4813 on petioles of *Cercropia* sp., Santa Rosa National Park). These litter collections were made in addition to our soil isolates and the soil isolates made near Guanacaste's provincial capital, Liberia, cited in Raper and Fennell (1965). This species was very difficult to identify from field collections, because only masses of Hülle cells were present. When cultured from field-collected Hülle cells, conidiophores rarely formed in agar culture. We were able to firmly establish the link among litter collections, soil isolates and the description in Raper and Fennell (1965) following the molecular analysis.

The abundance of sclerotium-forming and sexual species of Aspergilli in the soils an on fruits of the Guanacaste dry forest may be an adaptation to regularity of harsh dry seasons following luxuriant growth during the rainy seasons. Sclerotia or cleistothecia of Aspergilli form abundantly on or among the mycelia of vigorously growing laboratory cultures on agar media or on grains. It follows that sclerotium or cleistothecium formation on the surfaces of fruits would indicate that the mycelia of these fungi are actively colonizing and degrading the fruits (Wicklow, 1985). It would be interesting to measure how much these Aspergilli contribute to the rapid single season decomposition of these fruits. Presumably, sclerotia and ascospores from Aspergilli fall from

decomposed fruits and are reintroduced into the soil as the fruits disintegrate. The same Aspergilli observed on fruits also were abundant in the soil (Table 2). Another series of questions relates to how the toxic nature of *Aspergillus* sclerotia, mycelia, and extracellular metabolites are involved in the colonization of fallen fruits and the defense of vulnerable sclerotia and cleistothecia on fruit surfaces. Mycelia and sclerotia of Aspergilli produced in grain-based or other solid fermentation systems are prolific producers of secondary metabolites and these metabolites may accumulate in toxigenically significant quantities in infested substrata (Lindenfelser and Ciegler, 1975; Kobbe *et al.*, 1977; Singh *et al.*, 1991; Gloer, 1997). We believe these fruit-infesting populations in the dry forests of northwestern Costa Rica offer an excellent opportunity to explore the adaptations and evolution of toxigenic Aspergilli in a natural system.

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